Indolo[2,3-*b*]-Quinolizinium Bromide: An Efficient Intercalator with DNA-Photodamaging Properties

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The associative interactions of indolo[2,3-b]-quinolizinium bromide with DNA and its DNA photocleavage properties were studied in detail. Absorption and emission spectroscopy, linear dichroism, and energy-transfer measurements indicate that the indoloquinolizinium binds to DNA primarily by intercalation, with a preference for GC base pairs. In agreement with this data, the results of primer extension analysis indicate that photocleavage occurrs prevalently at the GC nucleotides. Molecular modeling studies confirm that intercalative stacking between adjacent base pairs is energetically favorable. However, it is also observed that the location of the dye in the minor groove of the DNA is energetically even more favorable. Upon UVA irradiation, the indoloquinolizinium causes single-strand cleavage with an efficiency that varies with the dye–DNA ratio. This observation is rationalized in terms of more efficient photocleavage by the externally bound dye compared with the intercalated one. The kinetics of strand degradation under aerobic and anaerobic conditions suggest that a Type I reaction occurs, that is, radical-mediated DNA damage.

KEYWORDS:

bioorganic chemistry · DNA cleavage · molecular modeling · photobiology · quinolizinium salts

Introduction

In recent years much interest has been focused on molecules that may bind and modify genetic material.[1–3] Along these lines, there has been increased interest in the discovery and investigation of compounds that cleave DNA when irradiated with visible or UV light. These compounds, called photonucleases,[3] exhibit a large potential for therapeutic applications because they are often inert until activated by light and permit control of the reaction both in a spatial and temporal sense. The photonucleases discovered so far operate by several distinct mechanisms. One class of compounds photosensitizes the excitation of reactive intermediates that react with DNA, such as singlet oxygen[4, 5] or the hydroxyl radical[6–8]. In a second class, the photonuclease is bound to the nucleic acid before its activation and the DNA damage is thus localized at or near the binding site.[9–11]

Photonucleases, like any other small DNA-binding molecule, associate by intercalation or fit into the minor groove of the DNA.[12] Importantly, the type and efficiency of the photocleavage reaction will depend on the binding site that the photonuclease occupies. Intercalators usually exhibit a planar structure with at least two annelated aromatic rings. In most cases, a positive charge is required for an appropriate binding affinity.[12] This cationic moiety is usually provided by a quaternary nitrogen atom, which may be generated either by simple protonation, as in the aminoacridine series (for example, proflavine), or by *N*-alkylation, as found in the well-known phenantridine series (for example, ethidium bromide). Alternatively, it is possible to introduce the cationic nitrogen atom as the bridgehead between two annelated aromatic rings, as in the benzo[*b*]quinolizinium salts **1a–c** (Scheme 1), coralyne (**2**), and related quinolizinium salts such as **3**, whose DNA-binding and DNA-photodamaging properties have already been shown.[13–18] Recently, we reported the synthesis of the novel compound indolo[2,3-*b*]-quinolizinium bromide (**4**) and showed in preliminary experiments that this compound binds to DNA and leads to photoinduced DNA damage.[19] The interactions of this aromatic dye with DNA seemed to be of special interest since it closely resembles the structure of the known antitumor drug Céliptium (**5**) and that of the antibacterial and antimalarial alkaloid cryptolepine (**6**). Herein, we present a detailed study of the binding interactions of the indoloquinolizinium derivative with DNA, along with the propensity of this compound to cause photoinduced DNA damage.

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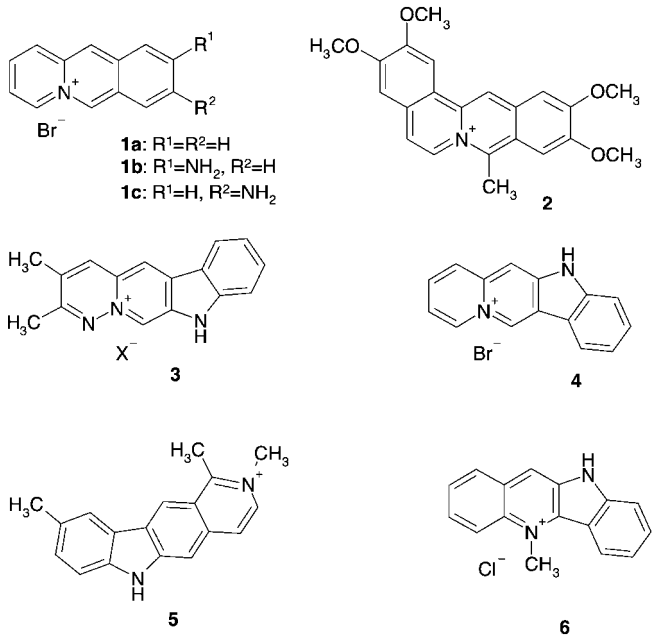

Scheme 1. *Cationic DNA intercalators.*

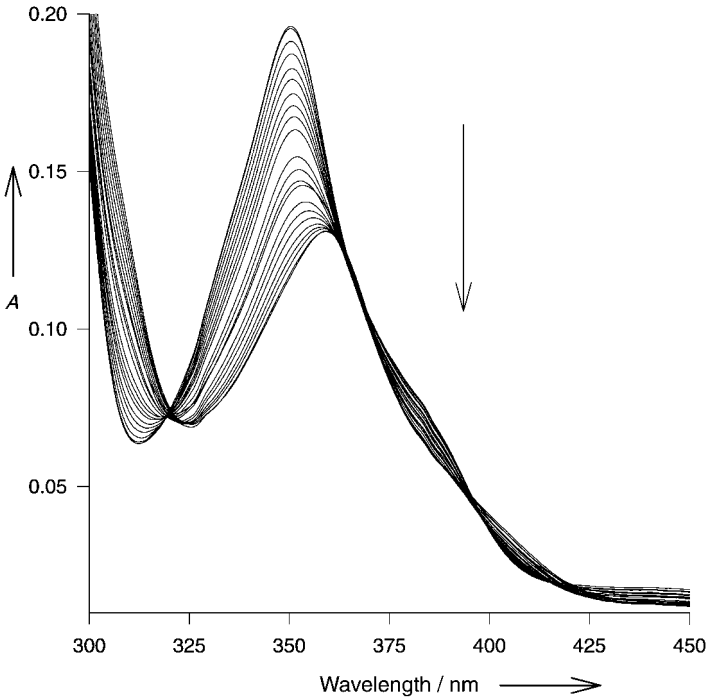

Figure 1. *Spectrophotometric titration of st-DNA to indoloquinolizinium **4** in ETN buffer (0.01 M; pH 7.0), T = 25 °C.*

Results

Spectrophotometric titrations

The binding of **4** to DNA in buffered aqueous solution was monitored by absorption and fluorescence spectroscopy. The absorption of the compound was recorded in the absence and in the presence of salmon testes DNA (st-DNA). The absorption maximum of quinolizinium salt **4** exhibits a bathochromic shift of about 10 nm in the presence of nucleic acids relative to the free dye **4** (Figure 1). The spectrophotometric titration data for **4** titrated with DNA at different salt concentrations were used to estimate binding constants as well as binding-site size according to the method of McGhee and von Hippel.[20] The corresponding binding isotherms, represented as a Scatchard plot,[21] are given in Figure 2 and the thermodynamic parameters, that is, binding constant K_i and binding-site size n are shown in Table 1. The DNA binding constants decrease as a function of Na^+ ion concentration. At the various salt concentrations the exclusion parameter n is almost constant and found to be equal to an average of two base pairs. By contrast, the binding constant K_i significantly depends on the concentration of Na^+ ions (Figure 2, inset), that is, the binding affinity decreases with increasing salt concentration. These data were analyzed according to the polyelectrolyte theory of Record et al.[22] Thus, the number of counterions released from the DNA upon binding of a charged molecule may be determined from the plot of log K_i versus log [Na^+], where the slope is $m\psi$ (Figure 2, inset). The parameter m represents the charge of the binding molecule and ψ is the fraction of counterions associated with each DNA phosphate moiety ($\psi = 0.88$ for double-stranded B-DNA[23]). An m value of approximately 0.7 was obtained for the indoloquinolizinium salt **4**. The

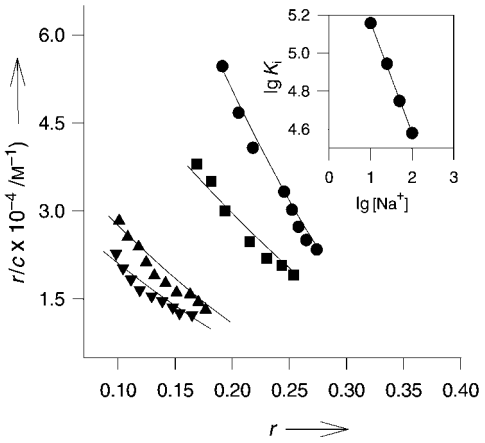

Figure 2. *Scatchard plots of spectrophotometric titrations of st-DNA to **4** in ETN buffer at different ionic strengths (● = 0.01 M, ■ = 0.025 M, ▲ = 0.050 M, ▼ = 0.1 M). The solid lines represent the best fit of the experimental data to the McGhee and von Hippel equation.[20] Inset: Plot of log K_i versus log [Na^+].*

Table 1. *Thermodynamic parameters determined by spectrophotometric titrations of st-DNA to **4** at different ionic strengths.*

[Na^+] [M][a]	K_i [$M^{-1} \times 10^5$]	n[b]
0.01	1.64 ± 0.20	2.60 ± 0.13
0.025	0.88 ± 0.06	2.57 ± 0.26
0.05	0.51 ± 0.05	3.00 ± 0.17
0.1	0.35 ± 0.03	3.31 ± 0.43

[a] In ETN buffer (pH 7.0). [b] In base pairs.[20]

dependence of the binding constant on the salt concentration allows the observed free energy of binding ΔG_{obs} to be partitioned into two energy contributions [Eq. (1)]; the "nonelectrostatic" contribution to the free energy of binding, ΔG_{npe}, provides a measure of the nonelectrostatic forces (hydrogen bonding, van der Waals interactions) that stabilize the DNA – ligand complex, and the polyelectrolyte contribution, ΔG_{pe} arises from coupled polyelectrolyte effects, which may be calculated according to Equation (2).

$$\Delta G_{obs} = -RT\ln K_i = \Delta G_{npe} + \Delta G_{pe} \quad (1)$$

$$\Delta G_{npe} = -RT(m\psi)\ln[Na^+] \quad (2)$$

The polyelectrolyte contribution is mostly determined by the release of condensed counterions from the DNA helix upon binding of a charged ligand. The observed free energy of binding of the indoloquinolizinium salt **4** at an ionic strength of 10 mM was determined to be $\Delta G_{obs} = -29.8\ kJ\,mol^{-1}$. According to Equations (1) and (2) the polyelectrolyte contribution ΔG_{pe} is $-7.8\ kJ\,mol^{-1}$ and ΔG_{npe} has a value of $-22.0\ kJ\,mol^{-1}$. Thus, the nonelectrostatic interactions between **4** and the DNA are more important than the ionic ones for the stabilization of the DNA – dye complex since they provide approximately 70% of the overall free energy of binding.

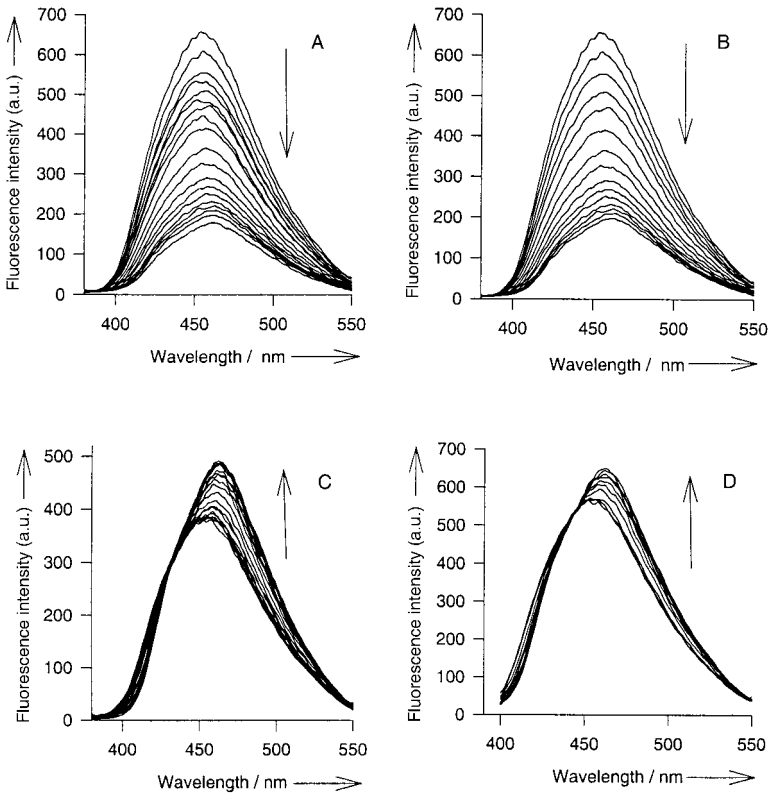

Figure 3. *Fluorometric titration of st-DNA (A), poly [dG-dC]$_2$ (B), poly[dA-dT]$_2$ (C), and poly[dI-dC]$_2$ (D) to dye* ***4*** *(5×10^{-6} M in ETN buffer (0.01 M; pH 7.0), T = 25 °C).*

Fluorometric titrations

Spectrofluorometric titrations of st-DNA and synthetic polynucleotides with quinolizinium salt **4** were also performed (Figure 3). The emission intensity of the free dye **4** was quenched upon addition of st-DNA. In particular, the addition of polydeoxyguanylic-deoxycytidylic acid sodium salt (poly[dG-dC]$_2$) to **4** was found to quench the fluorescence intensity significantly without a shift of the band maximum. By contrast, an increase of the fluorescence along with a shift of the emission maximum from 445 to 455 nm was observed on addition of polydeoxyadenylic-thymidylic acid sodium salt (poly[dA-dT]$_2$). Most notably, the binding of polydeoxyinosylic-deoxycytidylic acid sodium salt (poly[dI-dC]$_2$) to **4** is also accompanied by an increase of emission intensity and a bathochromic shift.

The fluorescence titration data were also analyzed according to the model of McGhee and von Hippel,[20] which was used to estimate the binding constant (K_i) and the number of base pairs, *n*, covered by one ligand (Table 2). For the titration with st-DNA, the observed constant ($1.5 \times 10^5\ L\,M^{-1}$) is in good agreement with the one obtained by spectrophotometric titrations ($1.6 \times 10^5\ L\,M^{-1}$, Table 1). The largest K_i value ($4.2 \times 10^5\ L\,M^{-1}$) was observed for binding of **4** to poly[dG-dC]$_2$, which indicates a pronounced affinity for this polynucleotide and suggests a preferred location for **4** at GC-rich regions of the DNA. Moreover, the exclusion parameter *n*, in other words, the binding site size, is about two base pairs for DNA and poly-[dG-dC]$_2$, which is in agreement with intercalation of the dye into the double strand of the polynucleotide. By contrast, this value is larger for poly[dA-dT]$_2$ and poly[dI-dC]$_2$, which is not consistent with an intercalative binding mode.

Table 2. *Thermodynamic parameters obtained by fluorometric titrations.*[a]

	$K_i \times 10^5$ [M^{-1}]	*n*[b]
st-DNA	1.47 ± 0.06	1.7 ± 0.03
poly[dG-dC]$_2$	4.20 ± 0.20	1.6 ± 0.05
poly[dA-dT]$_2$	1.24 ± 0.06	3.9 ± 0.02
poly[dI-dC]$_2$	1.48 ± 0.16	3.1 ± 0.02

[a] In ETN buffer (0.01 M, pH 7.0). [b] In base pairs.[20]

Flow linear dichroism

The linear dichroism (LD) spectra of **4** in the presence of DNA were also determined (Figure 4) since this methodology has been shown to be an efficient tool for evaluation of the distinct binding mode between a dye and nucleic acids.[24, 25] The LD signals of **4** is negative at all DNA – dye ratios, both in the UV region in which the DNA and the dye absorb, and at wavelengths where only the dye absorbs (Figure 4B). The LD signals in the long-wavelength absorption regions of the dye (300 – 400 nm) are negative in the presence of DNA, which indicates an induced orientation of the ligand chromophore upon binding to the DNA.[24, 25] A significant increase of the LD signal intensities in the absorption band of the DNA bases (260 nm) was also

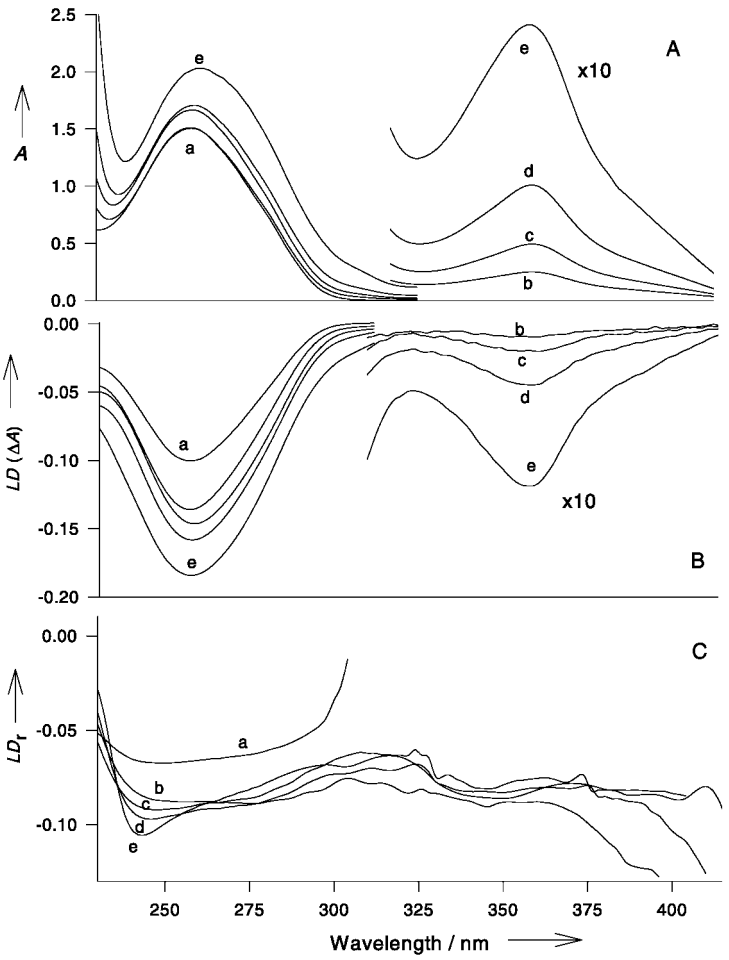

Figure 4. *Absorbance* A *(A), linear dichroism* LD *(B), and reduced linear dichroism* LD_r *(C) spectra of mixtures of st-DNA and* **4** *in ETN buffer (0.01 M; pH 7.0) at different dye – DNA ratios: a, 0.00; b, 0.02; c, 0.04; d, 0.08; e, 0.2.*

observed, which suggests that the ability of the DNA molecules to orient along the flow lines is increased upon binding to compound **4**.

The reduced LD spectrum (LD_r) provides further information on the average orientation of the transition moment of the dye relative to those of the DNA bases and allows homogeneous and heterogeneous binding to be distinguished. A nearly constant LD_r value was observed over the range 310 – 400 nm (Figure 4C), which unambiguously proves an almost exclusive intercalation of quinolizinium derivative **4** into the DNA.[24]

Fluorescence energy transfer

Fluorescence energy transfer experiments provide a complementary method in addition to linear dichroism spectroscopy for analysis of the binding mode of a dye with DNA.[26] In these experiments, the energy transfer from the excited DNA bases to a fluorescent ligand is monitored. Excitation energy transfer exhibits a distance dependence of R^{-6}, where R is the distance between the species involved in the transfer of energy. This distance dependence coupled with the relatively low fluorescence quantum yield of the DNA bases results in a short (4 – 7 Å) Förster critical distance R_0 at which half the energy of the excited DNA bases is transferred to the bound molecule.[27] Intercalation of the molecule between stacked bases within the DNA host duplex is associated with small host – guest distances (about 2 – 4 Å). Thus, the observation of efficient energy transfer from a host DNA to a bound molecule is consistent with an intercalative mode of binding. By contrast, binding of a molecule to the minor groove and/or dye stacking along the surface of the helix are associated with longer base – dye distances and minimal base – dye stacking interactions. Consequently, such binding modes are consistent with the absence of energy transfer from the host DNA to a bound dye. However, it should be noted that fluorescence energy transfer has been observed for some selected groove-bound molecules.[28]

Figure 5A shows curves that depict the wavelength dependence of Q_λ/Q_{310}. Q_λ is the fluorescence quantum yield of the complexes of indoloquinolizinium **4** with st-DNA, poly[dA-dT]$_2$, and poly[dG-dC]$_2$ relative to the fluorescence quantum yield of the free dye ($\lambda_{em} = 452$). The data were normalized with respect to 310 nm, a wavelength at which the DNA bases do not absorb. These plots show the wavelength of the absorbed photons which result in dye emission at the longest wavelength maximum and match very well the corresponding absorption maxima of the various DNAs (255 – 260 nm).

The experimental data were further analyzed with a plot of $F_B A_c/(1-10^{-A_c})A_B$ versus A_N/A_B (Figure 5B) in order to quantify the excitation energy transfer between the polynucleotides and **4**. According to Rayner et al.[29] this plot should be linear with a slope of $f\Phi_B\Phi_{ET}$ and an y-intercept at $f\Phi_B$. F_B is the excitation fluorescence intensity of the bound ligand, A_C, A_B, and A_N are the absorbances of the dye – DNA complex (C), the bound dye (B), and the DNA alone (N), Φ_B is the quantum yield of the bound dye, f is a correction factor that considers the differences in the incident light intensity, and Φ_{ET} is the quantum yield of the energy transfer. Figure 5B shows the plot of $F_B A_c/(1-10^{-A_c})A_B$ versus A_N/A_B for the complexes of **4** with st-DNA, Poly[dA-dT]$_2$, and Poly[dG-dC]$_2$ at a DNA – dye ratio of 10. All plots have positive slopes because Q_λ/Q_{310} is greater than one at wavelengths between 240 and 300 nm. This indicates that resonance energy transfer occurs from the nucleotide bases to **4** and is characteristic for intercalation. By contrast, a slope close to zero indicates the absence of energy transfer.

The quantum yield of the energy transfer, Φ_{ET}, may also be calculated by dividing the slope, $f\Phi_B\Phi_{ET}$, by the y-intercept, $f\Phi_B$. Moreover, the number of bases which are involved in the energy transfer, Φ'_{ET}, may be estimated from the product of the dye – DNA ratio and the Φ_{ET} value. This product, however, reflects the average number of bases that contribute to the transfer of energy, since not all donating bases contribute the same amount of energy. While the value of Φ_{ET} is proportional to the number of bound dye molecules, the product Φ'_{ET} is independent of this number and thus provides a useful measure of the energy transfer efficiency. The complex of **4** and poly[dG-dC]$_2$ has a significantly higher Φ_{ET} value (0.668) than the complexes with st-DNA (0.493) and poly[dA-dT]$_2$ (0.203). Moreover, the Φ'_{ET} data

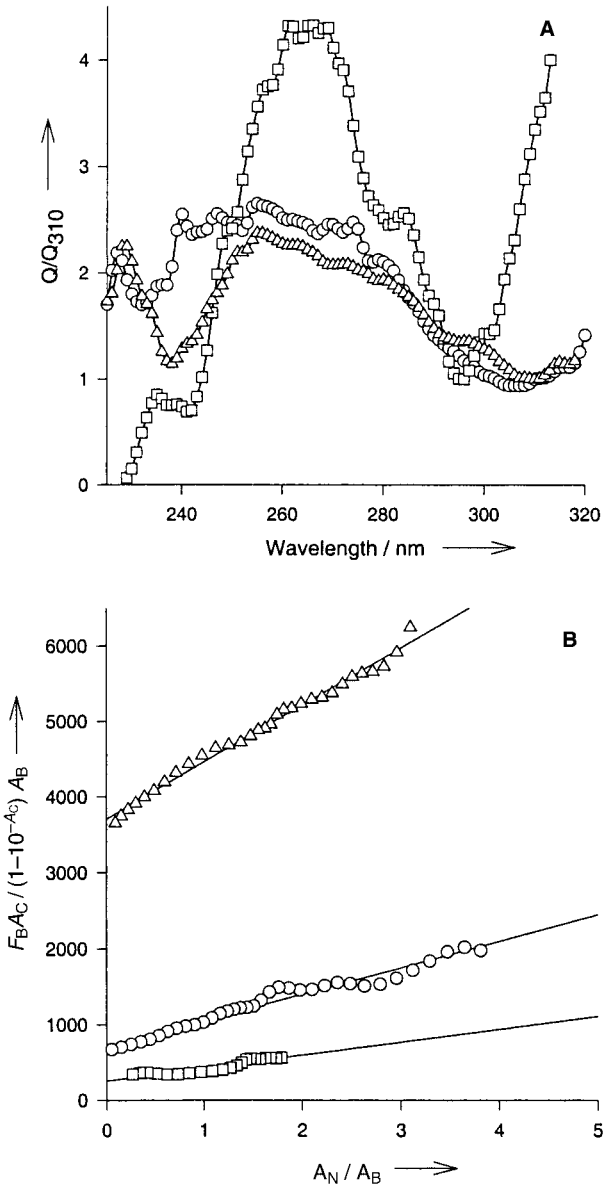

*Figure 5. Fluorescence energy transfer in complexes of DNA with **4**. A) Plots of Q/Q_{310} versus λ: st-DNA (○), poly[dG-dC]$_2$ (□), and poly[dA-dT]$_2$ (△); [dye]/[DNA] = 0.1; ETN buffer (0.01 M, pH 7.0); T = 25 °C. B) Plots of $F_BA_c/(1-10^{-A}c)A_B$ versus A_N/A_B: st-DNA (○), poly[dG-dC]$_2$ (□), and poly[dA-dT]$_2$ (△); [dye] – [DNA] = 0.1.*

reveal that in st-DNA 4.9 bases contribute to the overall energy transfer to **4**, whereas in the complex between **4** and poly[dG-dC]$_2$, 6.7 bases are involved in the energy transfer. In contrast, only 2 bases appear to participate in the energy transfer in the poly[dA-dT]$_2$ complex.

Molecular modeling studies

To estimate the structure of the complex formed between the indoloquinolizinium derivative **4** and DNA, a molecular modeling approach was used to explore energetically favorable structures that both complexes of **4** with poly[d(A-T)]$_2$ and with poly-[d(G-C)]$_2$ may adopt. The docking data for complexes of both DNA sequences show that the indoloquinolizinium **4** may adopt two significantly different binding motifs with duplex DNA. Figure 6 shows the complexes of **4** with poly[d(G-C)]$_2$. In the energetically more stable complex (with an energy minimum of

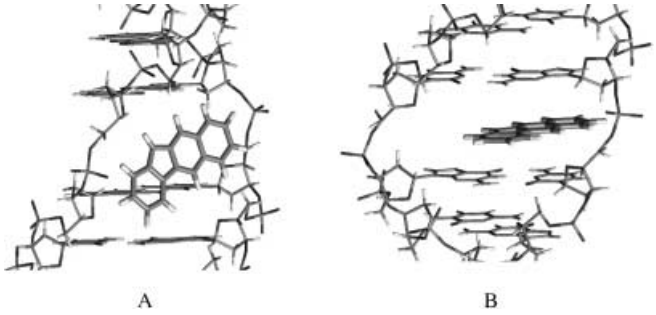

*Figure 6. The two structures of complexes between poly[d(G-C)]$_2$ and **4** with minimum energy as determined by molecular modeling: A) minor-groove binding; B) intercalation.*

about 180 kJ mol^{-1}) the dye molecule is located in the minor groove of the host duplex (Figure 6 A). In the less stable complex, the indoloquinolizinium intercalates into the host DNA. In this intercalation dye – DNA interaction, the planar indoloquinolizinium is stacked between adjacent DNA base pairs in an orientation that is almost perpendicular to the long axis of the DNA double helix. Moreover, the intercalation into G – C steps ($\Delta G = -64.7$ kJ mol^{-1}) is energetically more favorable than the one between A – T steps ($\Delta G = -39.3$ kJ mol^{-1}).

Photoinduced DNA cleavage

In order to investigate the DNA photocleavage activity of indoloquinolizinium **4**, it was irradiated in buffered aqueous solutions in the presence of supercoiled pBR322 plasmid DNA. The photosensitized DNA cleavage products were analyzed by agarose gel electrophoresis. In the dark, **4** does not promote DNA strand breaks (not shown). When the plasmid was irradiated in the presence of **4**, the DNA strand break was indicated by the formation of the relaxed, open circular plasmid form (not shown). The correlation of the DNA strand breaks with the irradiation time in the presence of **4** at different dye – DNA ratios is presented in Figure 7. At low dye – DNA ratios (0.01 and 0.04) at which the dye is intercalated in DNA no significant cleavage of the plasmid DNA was observed. As the dye – DNA ratio was increased (0.2, 0.4, and 0.8) and the dye became externally bound to the macromolecule, a significant nicking of the supercoiled plasmid form took place. To further evaluate the influence of reactive oxygen species on the photocleavage of the plasmid DNA, a series of experiments were carried out under anaerobic conditions (Figure 7). Removal of oxygen gas by bubbling nitrogen gas through the solutions did not reduce the cleavage rate, which indicates that reactive oxygen species, for example singlet oxygen, are not involved in the photoinduced DNA damage.

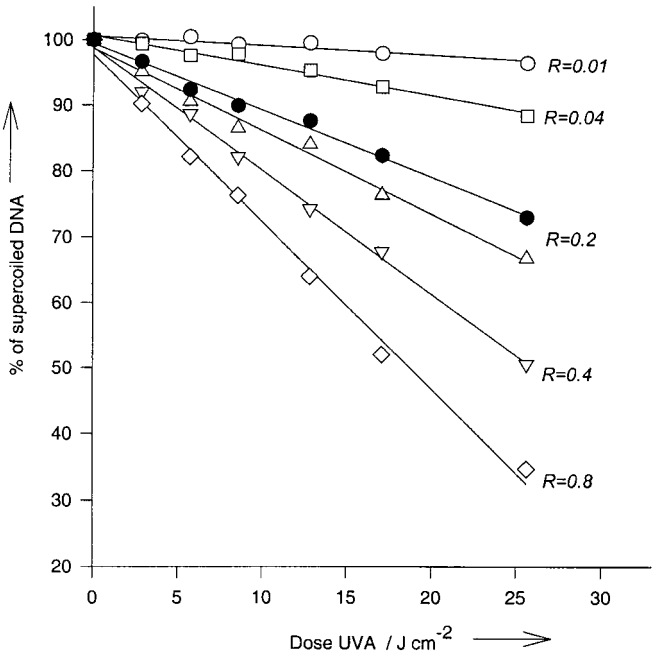

Figure 7. *Strand breaks of plasmid DNA (pBR322) on irradiation in the presence of dye* ***4*** *(λ = 365 nm) at the indicated dye – DNA ratios (R). The filled circles show the results of the experiments performed at a dye – DNA ratio of 0.2 in anaerobic conditions.*

Determination of the DNA photocleavage sites

To determine the sites of DNA breakage by the indoloquinolizinium **4**, pUC18 plasmid DNA was irradiated for 30 min and for 1 hour at molar dye – DNA ratios of 0.4 and 0.8 to generate partially cleaved DNA fragments (Figure 8). The photolysate was subsequently used for a primer extension analysis with the Taq polymerase and the M13 reverse primer. The method is based on the ability of the DNA polymerase to synthesize a complementary DNA strand by extension from the 5′-end labeled primer.[30] DNA synthesis by the Taq polymerase is arrested at the sites where the template strand is cleaved. Estimation of the length of the synthesized DNA fragments indicates the position of the photocleavage sites. To estimate the length of the labeled DNA fragments, they were separated by gel electrophoresis on a denaturing acrylamide gel, carried out along with the DNA sequencing reactions of the dideoxy nucleotide chain termination procedure.[31] The chain termination was performed on the same template by using cycle sequencing by the Taq polymerase primed with M13 reverse oligonucleotide. The results of the experiments are shown in Figure 8. The intensity of the DNA fragments, that is, the termination frequency, increases with longer irradiation times. Only minor spots were detected in a control reaction in the dark. An increase in the dye concentration (dye – DNA ratios = 0.4 and 0.8) also caused a moderate increase of the DNA photocleavage. Inspection of the autoradiogram indicated numerous termination sites with different intensities. Evaluation of the DNA cleavage sites in the examined sequence of 134 nucleotides (Figures 8 and 9), indicated a total of 67 break points, most of which occurred at guanine residues (45%). However, there were 23 intense bands, which correspond to the most frequent termination sites. These occurred prevalently at cytosine residues (9 break points, 39%) and at guanine residues (7 break points, 30%), whereas strand breaks at the adenine (4 break points, 17%) and thymine (3 break points, 14%) residues occur with a low frequency.

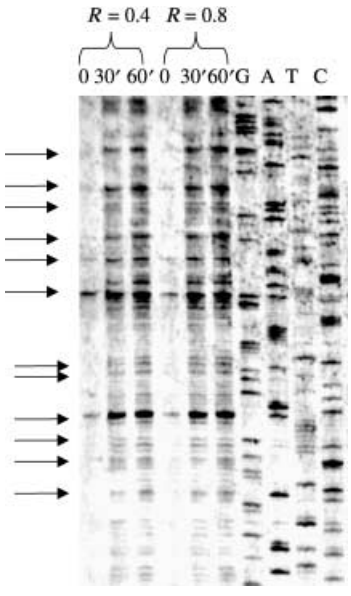

Figure 8. *Autoradiogram of 6% acrylamide/50% urea gel, which shows the DNA fragments synthesized from the M13 labeled primer by Taq DNA polymerase. The pUC18 template was irradiated in the presence of indoloquinolizinium salt for the indicated times (0, 30, 60 min) and at the indicated dye/DNA ratios (R = 0.4 and 0.8). The sequence of the same DNA fragment was run site by site as a reference (GATC). The major termination sites are indicated by arrows.*

5′GCATGCAAGCTTGGCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAA

AACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCC

AGCTGGCGTAATAGCGAAGAGGCCCGCACCGA

Figure 9. *The sequence of the synthesized DNA strand of the pUC18 plasmid is shown from position 275 to 407. All the termination sites are underlined. The nucleotides at which the polymerase acts more frequently are indicated in bold and double underlined.*

Discussion

The significant decrease in the absorbance and the quenching of the fluorescence intensity of indoloquinolizinium **4**, along with the bathochromic shifts of the absorption and emission maxima on addition of DNA, clearly indicate an associative interaction of **4** with the nucleic acids. The binding affinity of **4** for DNA is comparable to those of DNA-binding dyes with similar structures. In addition, the binding of the dye **4** was further confirmed by the appearance of induced circular dichroism in the presence of st-DNA and synthetic polynucleotides (see the Supporting Information). Moreover, the analysis of the fluorometric titrations reveals that the dye **4** binds to DNA with a significant preference for GC base pairs in the helix. Further studies showed that the binding affinity is mostly determined by nonionic interactions.

The quenching of the fluorescence intensity of **4** in the presence of DNA is most likely to be caused by an electron transfer reaction to the excited dye from the guanine bases in the helix as has already been shown for other dyes.[10, 32–34] This proposal was confirmed by the observation that the addition of poly[dG-dC]$_2$ to **4** resulted a significant emission quenching, whereas an increase of fluorescence intensity was observed on addition of poly[dA-dT]$_2$ and poly[dI-dC]$_2$. These results may be explained by the fact that guanine is the nucleic base with the lowest one-electron oxidation potential.[35] Apparently the oxidation potential of adenine and inosine is not low enough to allow an exergonic electron transfer from these bases to the excited dye **4**.[36] Therefore the emission intensity increases slightly on addition of poly[dA-dT]$_2$ and poly[dI-dC]$_2$, presumably because the binding to the DNA partially protects the dye from the solvent and thus suppresses deactivating dye – solvent interactions.

Isosbestic points in the spectrophotometric titrations indicate the presence of a single binding mode, in other words one type of quinolizinium – DNA complex is formed almost exclusively. The flow LD spectroscopic investigations of the association of **4** with DNA confirm this homogenous binding mode. As has been demonstrated for numerous other intercalating compounds, the negative phase of the LD signal of the dye and the wavelength independence of the LD absorption unambiguously demonstrate that the indoloquinolizinium **4** almost exclusively intercalates into the DNA helix. Also, the significant increase of the LD signal of the DNA bases is consistent with intercalation of **4**, which results in a more pronounced orientation of the DNA along the hydrodynamic field of the rotating cuvette caused by lengthening and stiffening of the helix upon intercalation. Complementary to the LD experiments, the fluorescence energy transfer experiments provide further evidence for an intercalation binding mode since it was shown that each excited nucleic base transfers its energy to the indoloquinolizinium salt **4**, which results in fluorescence of the dye. Such an energy transfer is proposed to indicate a mode of binding that involves intercalation. The data for the quantum yield of the electron transfer, Φ'_{ET} (Table 3), confirm the preference of **4** for binding at GC-rich regions in the DNA. These data show that in poly[dG-dC]$_2$ more nucleic bases contribute to the electron transfer than in poly[dA-dT]$_2$. These results also indicate that the energy transfer from the DNA bases of poly[dG-dC]$_2$ to the intercalated **4** involves the contribution of the nearest neighbor bases and of the flanking bases. Although it is demonstrated that **4** also intercalates between AT base pairs, it is only partially intercalated in poly[dA-dT]$_2$ so that only two bases are close enough to the dye to contribute to the energy transfer. In agreement with these results, the primer extension analysis revealed that the indoloquinolizinium **4** exhibits sequence selectivity in DNA photocleavage with a preference for GC-rich tracts.

Table 3. *Quantum yields of energy transfer from st-DNA and synthetic polynucleotides to the bound indoloquinolizinium salt **4**.*

	Φ_{ET}	Φ'_{ET}
st-DNA[a]	0.493	4.93
Poly[dG-dC]$_2$[a]	0.668	6.69
Poly[dA-dT]$_2$[a]	0.203	2.03

[a] In ETN buffer (0.01 M, pH 7.0); [DNA]/[**4**] = 10.

Interestingly, the modeling studies showed that binding of indoloquinolizinium **4** to the minor groove of DNA is energetically more favorable than intercalation by about 18 kJ mol^{-1}. However, since these data only consider the energy of the complexes without the hypersurface of the complete reaction pathway, it may be that the groove binding has a larger activation energy than the intercalation. This kinetic control of complex formation may result from the fact that prior to groove binding of the dye, condensed cations (for example, Na^+, K^+) have to be released from the DNA backbone, which results in a relatively high activation barrier. It should also be noted that as a result of attractive electrostatic interactions between the phosphate moieties in the minor groove and positively charged ions, a complex of DNA with any cationic molecule in the minor groove will be energetically relatively more stable. By contrast, the observation of an energetically favorable intercalation by the molecular modeling method is relatively unusual.[37, 38] Thus, these calculations reveal that intercalation of indoloquinolizinium **4** is indeed an energetically favorable process, which is in good agreement with the experimental results.

The DNA cleavage may not be exclusively initiated by the intercalated molecules, rather the DNA damage is most likely to be caused by the initial abstraction of a hydrogen atom from the deoxyribose. It may therefore be assumed that a favorable position for a dye to initiate this reaction is at the backbone rather than inside the DNA. Thus, excited molecules that approach the DNA backbone by collision or the few molecules that bind to the minor groove may react more efficiently than the intercalated ones. It was demonstrated that indoloquinolizinium **4** may lead to photoinduced DNA damage, which depends on the reaction time and on the dye concentration. Since the efficiency of the DNA damage is not significantly influenced by the presence of oxygen, a mechanism that involves the generation of singlet oxygen may be excluded. No alkaline work-up of the reaction mixture is required to detect the strand breaks, thus the DNA damage is likely to be introduced by hydrogen abstraction at the deoxyribose moiety of the DNA, for example, according to the mechanism proposed by Schulte-Frohlinde et al.[39] and further elucidated by Giese et al.[40] An alternative pathway involves oxidative transformations of the DNA bases by an intercalated dye. However, in this case alkaline work-up is usually necessary to detect DNA strand breaks in the pBR322 assay.

In summary, a detailed analysis of the binding properties of indoloquinolizinium **4** with DNA is presented that reveals a strong and almost exclusive intercalation interaction. Thus, the indolo[2,3-*b*]-quinolizinium may represent a novel promising platform for the design of DNA-binding molecules with even higher binding affinities and selectivities. Indoloquinolizinium **4** also exhibits DNA-photodamaging properties. However, the selectivity of this process remains to be improved.

Experimental Section

Materials: Indolo[2,3-*b*]-quinolizinium bromide (**4**) was synthesized according to published procedure.[19] Salmon testes DNA sodium salt and poly[dA-dT]$_2$ were purchased from Sigma (Saint Louis, MI, USA). Poly[dG-dC]$_2$ and poly[dI-dC]$_2$ were purchased from Pharmacia and used without further purification. The actual concentrations (in nucleotides) were determined by UV spectroscopy (st-DNA and [poly(dAdT)]$_2$, $\varepsilon_{260} = 6600\,\text{M}^{-1}\text{cm}^{-1}$; [poly(dGdC)]$_2$, $\varepsilon_{254} = 8400\,\text{M}^{-1}\text{cm}^{-1}$; [poly(dIdC)]$_2$, $\varepsilon_{251} = 6900\,\text{M}^{-1}\text{cm}^{-1}$). All other reagents were of analytical grade.

DNA binding studies: Nucleic acid concentrations, expressed with respect to mononucleotides, were determined spectrophotometrically by using the reported data for the molar absorption coefficient at the wavelength indicated in the text. UV/Vis absorption spectra were recorded on a Perkin-Elmer Lambda 15 spectrophotometer and fluorescence spectra were recorded on a Perkin-Elmer LS-50B luminescence spectrophotometer. All measurements were carried out in ETN buffer (tris(hydroxymethyl)aminomethane (Tris; 10 mM), ethylenediaminetetraacetate (EDTA; 1 mM), NaCl to adjust the ionic strength to the desired values) at pH 7.0 and 25 °C. The titration experiments were performed according to published procedures[17] and the data, plotted as a Scatchard plot,[21] were analyzed by the method of McGhee and von Hippel.[20] to obtain the intrinsic binding constant (K_i) and the binding site size (n).

Flow linear dichroism: LD spectra were recorded in a flow cell on a Jasco J500A spectropolarimeter equipped with an IBM PC and a Jasco J interface. The determination and interpretation of the data was performed according to a previous publication.[17]

Fluorescence contact energy transfer: Contact energy transfer from DNA bases to the associated dye was determined from the excitation spectra of the DNA – dye complex from 230 to 330 nm with an interval of 1 nm. Fluorescence was monitored at $\lambda_{em} = 452$ nm. The excitation spectra were corrected for the inner filter effect prior to normalization.[41] The respective quantum yields Q were determined according to Equation (3).

$$Q = q_b/q_f = I_b E_f / I_f E_b \tag{3}$$

The parameters q_b and q_f represent the fluorescent quantum efficiencies of bound and free dye, and I_b and I_f are the fluorescence intensities of the dye in the presence and absence of DNA, respectively. E_b and E_f are the corresponding molar extinction coefficients of the dye. The term Q_λ/Q_{310} was plotted versus the wavelength.

In order to quantify the excitation energy transfer between DNA and **4**, the experimental data were used to produce a plot of $F_B A_c/(1-10^{-A_c})A_B$ versus A_N/A_B. The plot was analyzed according to Equation (4).[29]

$$F_B A_c/(1-10^{-A_c})A_B = f\Phi_B\Phi_{ET}(A_N/A_B) + f\Phi_B \tag{4}$$

The symbols in this equation are defined in the text.

Computational methodologies: Calculations were performed on a Silicon Graphics Octane R12000 workstation. This study involved the use of consensus dinucleotide intercalation geometries d(ApT) and d(GpC) initially obtained by using the NAMOT2 (Nucleic Acid Modeling Tool, Los Alamos National Laboratory, Los Alamos New Mexico) software.[42]The d(ApT) and d(GpC) intercalation sites were contained in the centre of a decanucleotide duplex and had the sequences d(5′-ATATA-3′)$_2$ and d(5′-GCGCG-3′)$_2$, respectively. Decamers in B-form were built by using the "DNA Builder" module of the Molecular Operating Environment (MOE 2001.01) software.[43] Decanucleotides were minimized with an Amber94 all-atom force field,[44] implemented by the MOE modeling package, until the root mean square (rms) value of the truncated Newton method (TN) was less than 0.001 kcal mol^{-1} Å^{-1}. The dielectric constant was assumed to be distance independent with a magnitude of 4. The ground state geometry of the indoloquinolizinium derivative was fully optimized without geometry constraints by using RHF/3 – 21G(*) ab initio calculations. Vibrational frequency analysis was used to characterize the minima stationary points (zero imaginary frequencies). The software package Gaussian98 was utilized for all quantum mechanical calculations.[45] The indoloquinolizinium **4** was docked into both intercalation sites by using flexible MOE-Dock methodology. The purpose of the MOE-Dock method is to search for favorable binding configurations for a small, flexible ligand with a rigid macromolecular target. The search is conducted within a user-specified 3D docking box with a simulated annealing protocol and an MMFF94 force field. The MOE-Dock program performs a user-specified number of independent docking runs (55 in our case) and writes the resulting conformations and their energies to a molecular database file. The resulting DNA – dye intercalated complexes were subjected to Amber94 all-atom energy minimization until the rms of the conjugate gradient was less than 0.1 kcal mol^{-1} Å^{-1}. Charges for the ligands were imported from the Gaussian output files. To model the effects of solvent more directly, a set of electrostatic interaction corrections were used. The MOE program suite implemented a modified version of the generalized Born/surface area contact function described by Still and co-authors.[46] These terms model the electrostatic contribution to the free energy of solvation in a continuum solvent model. The interaction energy values were calculated as the energy of the complex minus the energy of the ligand, minus the energy of the DNA, as shown in Equation (5).

$$\Delta E_{inter} = E_{(complex)} - (E_{(L)} + E_{(rDNA)}) \tag{5}$$

DNA photocleavage experiments: Irradiation was performed with a HPW 125 Philips lamp (365 nm, 0.230 J cm^{-2} min^{-1}) in a quartz cuvette (0.1 cm path length) fitted with a plastic stopper through which a venting needle was inserted for nitrogen purging. The irradiated samples contained pBR322 DNA (20 mg L^{-1}) dissolved in ETN buffer (10 mm; pH 7.0) and the examined compounds. After different irradiation intervals, the solution (5 μL) was removed and diluted with the loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, and 30% w/v glycerol in water) then the samples were loaded on 1% agarose gel. The electrophoretic run was carried out in TAE buffer (Tris-acetate (0.04 M), EDTA (1 mM)) at 50 V for 4 hrs by using the GNA-100 electrophoretic apparatus (Pharmacia, Uppsala, Sweden). After staining in ethidium bromide solution (1 μg mL^{-1} in TAE buffer) for 20 min, the gel was washed with water and the DNA bands were detected under UV radiation with a UV transilluminator. Photographs were taken with a digital photocamera Kodak DC256 and the quantification of the spots was achieved by the image analyzer software Quantity One (BIO RAD, Milano Italy). The fraction of supercoiled DNA was calculated as described by Ciulla et al.[47]

Primer extension and DNA sequencing: Plasmid pUC18 was purified from transformed *Escherishia coli* strain HB101 by the GeneElute plasmid miniprep kit (Sigma Saint Louis, MI USA) and stored at 4 °C in TE buffer (Tris-HCl (10 mM), EDTA (1 mM); pH 8). Plasmid DNA (50 μg mL^{-1}) was treated with indolo[2,3-*b*]quinolizinium bromide (**4**) at molar DNA ratios of 0.4 and 0.8 in 50 μL of ETN (0.01 M). The plasmid DNA was then irradiated with a UVA lamp (365 nm; energy emission 0.2848 J^{cm-2} min^{-1}). Aliquots were taken

after 0, 30, 60, and 90 min and kept on ice. Primer extension analysis was performed on the irradiated DNA (2.5 μL) in a 10 μL reaction volume that contained deoxynucleotides triphosphates (200 μM each, Amersham Pharmacia Biotech, Little Chaffold, UK), Taq polymerase (2 U, Roche Diagnostic, Mannheim, Germany), and the supplied buffer with magnesium chloride and the lacZ reverse primer M13, which was previously end-labeled with [γ-^{33}P] adenosine triphosphate following the instructions of the fmol-DNA sequencing kit (Promega Corp. Madison WI, USA). This method was also used to sequence the pUC18 plasmid, which was used as a reference to determine the position at which DNA photocleavage occurred. For primer extention and cycle sequencing, thirty PCR cycles were programmed with the sequence, denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, and extension for 1 min at 72 °C. Aliquots (1 μL) were loaded onto a denaturing 6% acrylamide, 50% urea gel and separated by gel electrophoresis. Samples were subsequently dried and exposed to Kodak X-OMAT UV films.

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